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REVIEW ARTICLE

New Methods for Clinical Proteomics in Allergy

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ABSTRACT

Recent genomic studies have revealed many kinds of genetic polymorphisms. Some genetic polymorphisms have a correlation with allergic phenotypes, however there is only a statistical association without a precise molecular mechanism being demonstrated. Analysis of the molecular mechanisms from a proteomic perspective should contribute to a better understanding of diseases and indicate possible therapeutic approaches. Recent advances in identification and characterization of many immunological molecules have led to a shift to profiling research, clinical proteomics, of already known factors. However, analysis of such biomarkers in allergies requires methodological improvements because allergic reactions can be greatly influenced by subtle changes of factors. These subtle changes cannot be detected by conventional techniques such as 2D-PAGE, and the grammar behind the system is not well recognized by conventional proteomics. Examples of innovative methods useful for proteomic approaches to allergies are discussed here ; especially high throughput screening and structural methods for allergy targeting.

KEY WORDS

clinical proteomics, high throughput screening, NMR, protein structure, therapeutic approach

INTRODUCTION

Recent genomic studies have revealed many kinds of genetic polymorphisms, some of them having a correlation with allergic phenotypes. However, the findings still remain basically statistical associations without precise molecular mechanisms being demonstrated. Analysis of the molecular mechanisms between the genotype and clinical symptoms should contribute to better understandings of diseases and indicate possible therapeutic approaches.

Several proteomic approaches have been described in association with allergic diseases. One is an animal experiment which found proteins closely related with allergic airway inflammation, and another is two-dimensional (2D)-PAGE analysis of allergens.[1-3] 2D-PAGE analysis can indicate new possible allergens associated with food allergies, which have not been characterized by conventional 1D-PAGE. Such findings by proteomics in allergy research would clarify the molecular mechanism of the allergic reaction with findings by genomics.

The word, proteomics, usually indicates searches for understanding of the grammar behind biological systems as a whole. However, recent advances in identification and characterization of many immunological molecules have led to shift toward profiling research of already known factors such as cytokines.[4-6] Allergic reactions are mediated by many kinds of factors. The specificity of allergic reactions is regulated by recognition in the HLA : peptide and TCR with many other factors subsequently modulating the responses. Many studies have shown that cytokines participate in the induction and effecter phases of inflammatory responses in allergies. Studies of the cytokine network are important for ascertaining candidates for drugs or as drug targets. Hence, measurements of these cytokines are widely used for monitoring immune responses in allergic diseases.[7,8]

Clinical proteomics focuses on the study to elucidate biomarkers of disease, however, analysis of such biomarkers in allergy requires innovative methodological improvements because allergies can be influenced by subtle changes of factors that cannot be detected by conventional techniques such as 2D-PAGE. Furthermore, the grammar behind the systems cannot be easily recognized by conventional proteomics.

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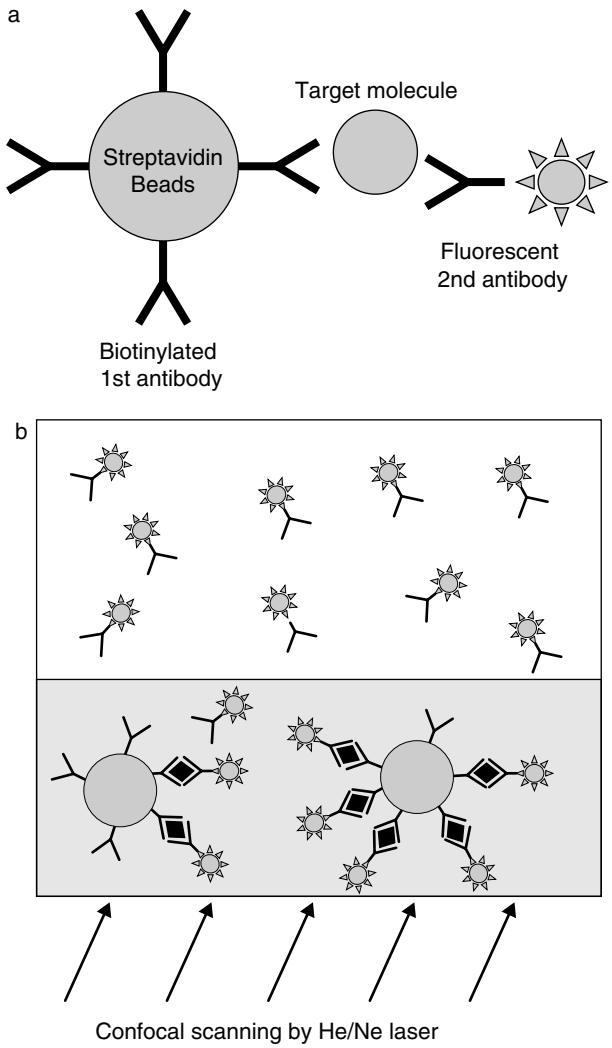

Fig. 1 Homogeneous assay of FLISA. **a**. Capture-antibody-coated beads and secondary detection antibody with fluorescence. **b**. A macro confocal imaging system with a helium/neon laser focuses on and scans fluorescent-bound beads resting on the bottoms of multiwell trays. Background fluorescence is minimal in relation to bead-bound fluorescence.

In the following we discuss examples of novel methods useful for proteomic approaches to allergies ; especially high throughput screening (HTS) and structural methods for allergy targeting.

HIGH THROUGHPUT SCREENING METHOD

Enzyme-linked immunosorbent assay (ELISA) is an established immunoassay used to quantify various cytokines. However, conventional ELISA, is not readily amenable for screening the large numbers of samples required for proteomic screenings due to the fact that it requires many processes and large quantities of antibody. For proteomic research, high throughput screening would benefit from an immunoassay that requires minimal manipulation and uses only a small amount of the compounds for screening. One such ELISA based method, beads-based cytokine measurement, has been developed and used for profiling of allergic states.[9]

In addition to ELISA-based methods, fluorescence-linked immunosorbent assay (FLISA) has been established as a method for HTS.[10] This system enables high throughput measurements and could be applied to the assessment of various factors such as cytokines or chemical mediators in allergic responses. A typical FLISA method involves the preparation of antibody-coated beads using streptavidin beads diluted in phosphate-buffered saline. A portion of the biotinylated anti-factor X monoclonal antibody (mAb) is added to the streptavidin beads solution and the uncaptured antibodies removed. Fluorometric microvolume immunoassay for factor-X is performed with an aliquot of samples placed into a 96-well plate. An aliquot of the beads mixture (antibody beads) is conjugated with anti-factor-X mAb. Appropriate buffer is then mixed with the sample or standard in each well and incubated for several hours at room temperature in the dark. After incubation, the 96-well plate is scanned using a macro confocal imaging system (Fig. 1). The average fluorescence per bead is recorded. A fluorescent microvolume assay technology instrument consists of a detector integrated with a bar code reader and a robotic plate handler that can accommodate large amounts of samples. A helium/neon laser is used to map the topology of the microplate bottom, and performs 256 scans across an area 1 mm × 1 mm × 100 mm deep. The emitted fluorescence passes through the same optical path as the excitation beam and a dichroic beam splitter for detection by photomultiplier tubes through filters.

The FLISA method is similar to a conventional ELISA method, but has several advantages. ELISA requires many incubation and wash steps, and also requires large amount of reagents such as monoclonal antibodies , making it practically unsuitable for screening large numbers of samples. In addition to this problem, ELISA can be influenced by temperature gradients in the microtiter plates. Studies using microtitration plates have demonstrated that a temperature gradient could exist between peripheral and center wells of an ELISA plate ; a gradient that may cause differences in an enzyme reaction between peripheral and center wells using microtitration plates, the so called edge-effect.[11,12] Samples must be handled carefully in ELISA to prevent edge-effects , whereas, FLISA has no enzyme reaction at the calorimetric phase, involving only the binding reaction of the antibody, so that edge-effects should be smaller in FLISA than ELISA. We obtained previous data which found FLISA to be more reliable than ELISA, and also that accurate screenings were very impor-

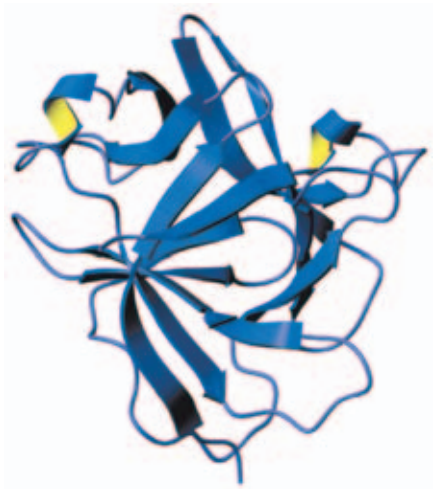

Fig. 2 Structure of human IL-18. Schematic ribbon drawing of the NMR structure of IL-18.

tant in proteomics, especially for drug discoveries.[13,14]

To quantify the many factors in the proteomes of different types of samples, FLISA is a more efficient assay method than a conventional ELISA which involves multiple incubation and washing steps, and requires large amounts of antibodies. The FLISA method is a homogeneous bead-based immunoassay that requires no wash steps. Over 100-fold less captured antibody is required in FLISA than in a conventional ELISA. However, the most striking difference between the two methods is the time required for the assay. Occupation time of a typical FLISA is about 1 hour, whereas a conventional ELISA will require several more hours.[15] A minimal time requirement is a crucial factor for applications such as HTS in proteomic research in allergology. FLISA is thus an attractive method as it involves less hands-on time and lower running costs.[15]

In immunological studies using FLISA, measurements of interleukins and lymphocyte count in whole blood have been reported.[10,16] The beads-based system of the FLISA is readily applicable to other plate-based assays, such as non-radioactive kinase, phosphatase, and protease assays. A multiplexed bead-based receptor-ligand binding assay has already been demonstrated using FLISA.[17,18] In addition, FLISA is capable of detecting and quantifying fluorescence on living cells, allowing for such diverse assays as apoptosis and cytotoxicity, and cellular immunoassays, and receptor ligand binding assays.[17-20] The FLISA system can be adapted for HTS of large libraries of proteomes, thus this system has a place in laboratories that routinely perform multiple, repetitive assays.

STRUCTURAL APPROACHES

Immunological reactions such as production of IgE are controlled by Th1 and Th2 cytokines. Disease specific mutations for IgE suppressive factors such as IL-12, IL-18, and IFN-gamma have been revealed.[6,21,22] For example, we previously studied IFN-gamma production from peripheral blood mononuclear cells of patients with asthma or dermatitis.[23] In most patients, both IL-18 and IL-12 induced the same magnitude of IFN-gamma production. However, in some patients, IL-18 induced only a small amount of IFN-gamma, while it was normal with IL-12.

In patients whose IFN gamma production with IL-18 is quite low, we found one amino acid deletion of the receptor. Predominant expression of the deletion of IL-18R alpha was associated with reduced IFN-gamma production by IL-18 but not IL-12, and was also associated with high serum IgE levels in children.[24] The actual effect of this deletion remains to be clarified by further analyses of the system.

For analysis of the mechanisms, we used structural biology techniques using multinuclear and multidimensional NMR. NMR analyses were performed using 1D, 2D, 3D, and 4D NMR, and structural calculations and refinements revealed the structure of IL-18 at atomic resolution (Fig. 2).[25]

Sample preparation is usually performed with recombinant protein, which is produced in a heterologous organism such as *E. coli* [26,27] For example, the 15N-labeled and 15N-13C-labeled wild-type proteins are expressed as a GST-fusion protein. Following purification by affinity chromatography, the GST tag is removed by digestion with proteases. Samples for NMR measurements typically consisted of 1−2 mM protein in buffers with neutral pH and adequate salt, in H2O/2H2O or 2H2O.

NMR analyses are performed using high-field magnetic spectroscopy.[28-32] NMR spectra can be acquired at approximately 310K on spectroscopy with a magnetic field such as 800 MHz NMR spectrometer. For assignment of the 1H, 13C and 15N resonances of the backbone and the side-chains, a series of three-dimensional experiments are performed. The stereospecific assignment of methyl groups of the Val and Leu residues was carried out. Distance restraints are obtained from 15N, 15N-, 15N, 13C- or 13C, 13C-resolved 4D NOESY experiments with an appropriate mixing time.

Structural calculations were performed as the third step. Initially, structure calculation and NOE peak assignment were performed in an iterative and manual manner using a specific program for calculation.[33-38] Backbone torsion angle restraints were derived from 3JHNHa of HNHA. The torsion angles c1 of phenylalanine, tyrosine, and histidine, were estimated from 3JC′C and 3JNC coupling constants. After determining the global fold manually, an automated algorithm can be used for the assignment of the remaining NOE peaks, yielding meaningful NOE upper distance

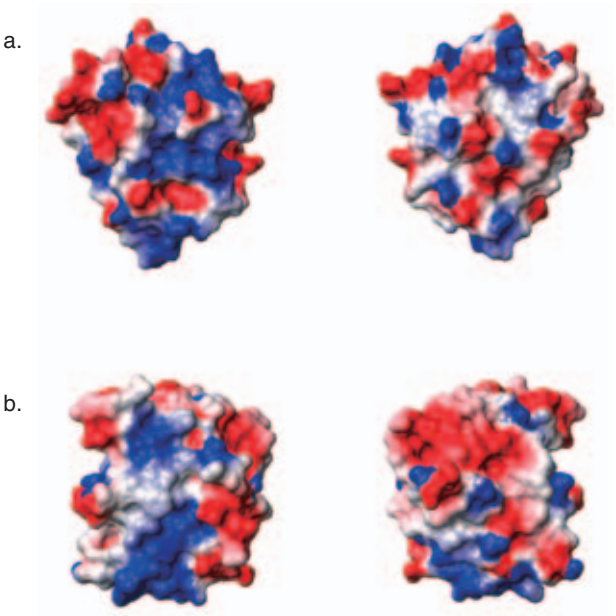

Fig. 3 Distribution of the electrostatic potential on the solvent-accessible surface. Blue corresponds to positive potential and red to negative potential. **a**, The IL-18 molecular is shown in the same orientation as for Figure 2 (left) and rotated by 180 degrees about the vertical axis (right). **b**, Distribution of the electrostatic potential on the solvent-accessible surface of IL-1beta.

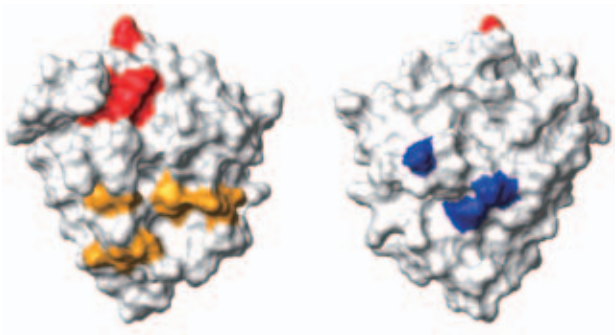

Fig. 4 Surface representation of IL-18 residues, the mutations of which resulted in a substantial reduction in the activity. Residues in sites I, II and III are in red, orange and blue, respectively.

restraints. With these restraints, final structures were calculated using the refinement program. At this stage, hydrogen bond restraints from the slowly exchanging backbone amides can be added as distance restraints for N-O and for HN-O atom pairs respectively. Nonstereospecifically assigned protons were treated as a floating chirality. Usually, a total of 100 structures were refined and the 20 lowest energy structures were analyzed using structure verification software.

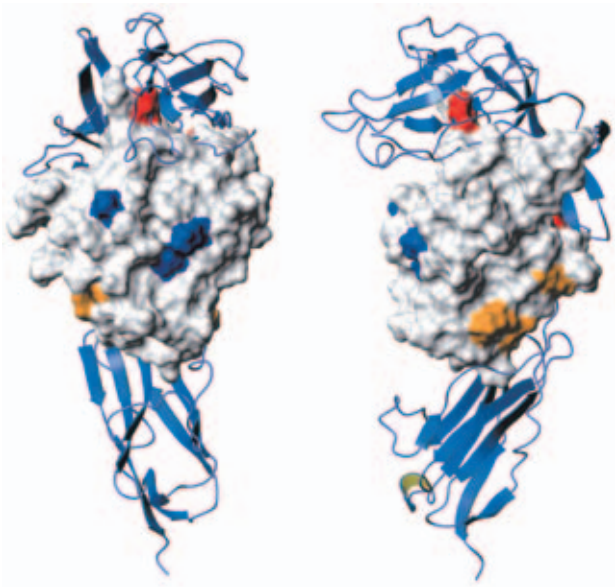

Fig. 5 Structure of the IL-18 : IL-18R alpha complex. IL-18 residues in sites I, II and III are in red, orange and blue, respectively.

The determined IL-18 structure has the same protein folding as that of IL-1, but IL-18 is distinctively different from IL-1 in its functional properties.[25,39-44] This means that the presence of these structural differences reflects their functional differences. Structural comparison between the two showed relatively large differences in three parts. Although the folding of the proteins were quite similar, distribution of the electrostatic surface potentials between the two were very different (Fig. 3). These differences at an atomic level serve as the factors for molecular discrimination.

When we mapped the critical residues for the biological activity on the surface of the structure, we found that they formed three distinct sites, named as sites 1, 2, and 3 (Fig. 4). From other experimental findings such as protein-protein interactions, the ligand, IL-18, was seen to bind to IL-18Ralpha using sites 1 and 2, but not site 3.[25,45-51] When we viewed its 3-D structure, we could see that sites 1 and 2 were covered by a receptor with atomic interactions, whereas site 3 was completely free (Fig. 5).

Previously reported genetic polymorphisms are not involved within the three sites. One of the identified coding SNPs, R210H, had interactions with the ligand, IL-18, suggesting an effect for binding affinity.[25,52] The deletion of alanine 317 closely associates with the functional abnormality in IL-18 signaling.[24,25] Our modeling study indicates that the deletion should not change the overall structure, but make the link shorter at a point just before the transmembrane domain by approximately five angstroms.

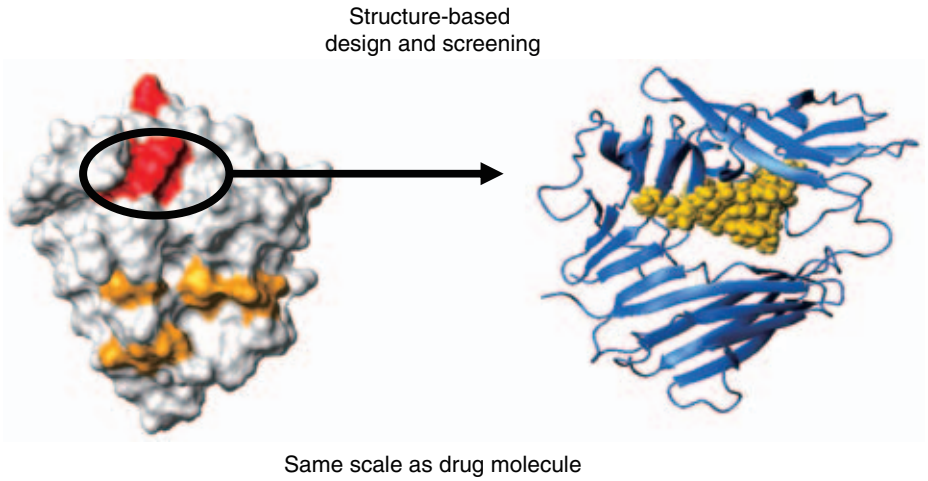

Fig. 7 Atomic scale strategy for proteomics toward therapeutic approaches against allergy.

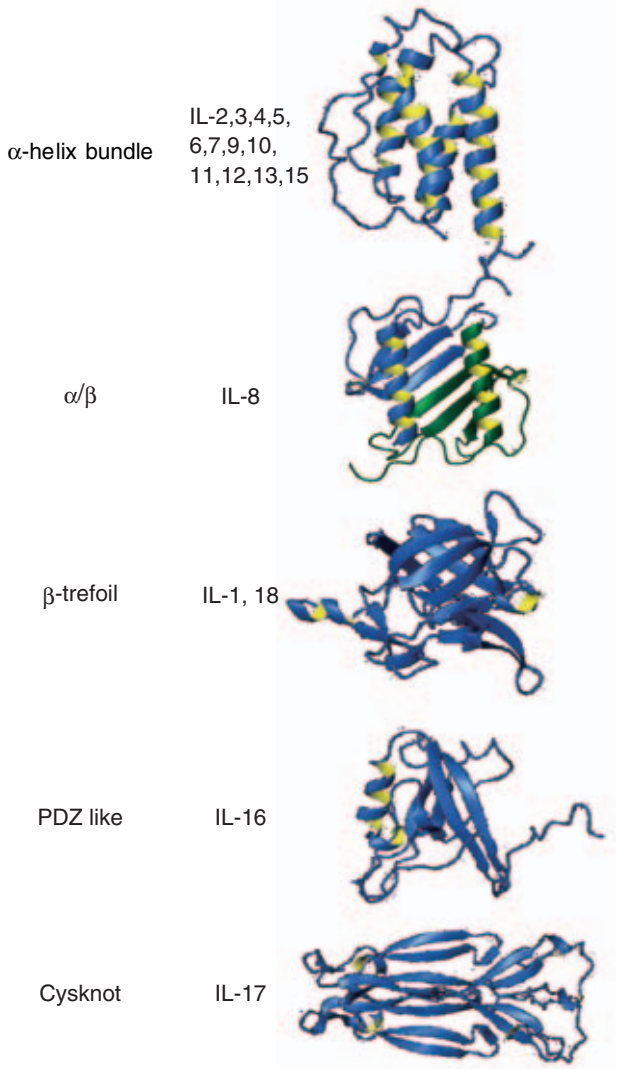

Fig. 6 New classifications for interleukins. The major group is alpha helix bundle types, with IL-8 a member of the chemokines from its structural properties in addition to its functional properties. The new class is the beta-trefoil group, and there are several orphan classes.

Taken together with other experimental data, we could ascertain that a tertiary complex formation could be achieved by interactions with receptor alpha at sites 1 and 2. For receptor beta, site 3 and additional interactions between receptors would be involved.[25,51] The deletion should effect the signal transduction modifying the alignment of the intracellular domains. Other SNPs on 232 can also have a functional change on their receptor- receptor interactions.[25,51,52]

From these data, we can now classify interleukins by their structures (Fig. 6). Before our structural determination of IL-18, several structures had been revealed. Our study proposed a new structural class, beta- trefoil interleukins, IL-1 and IL-18. The additional structural investigations by our group and other research groups have shown a catalogue of interleukins. From these data, we have postulated a new classification for interleukins. The major group is alpha helix bundle types, with IL-8 being a member of the chemokines which is based on its structural properties in addition to its functional properties. The new class is the beta-trefoil group also in addition to other orphan classes.[53-55]

TOWARD THERAPEUTIC APPROACHES

From a therapeutic aspect, the atomic interactions between ligand and receptors should present a target for drugs. For example, in this IL-18 system, the overall binding surface is too large to be a target, but for example, site 1 residue aspartic acid has a critical force for binding.[25] It has a complementary charge interaction with arginine of the receptor residue. The alanine mutant protein showed a significant loss of biological and receptor binding activities. This mutation was shown to have an effect related to the reduction of local surface negative potential, but not in other regions. Thus, we can focus on a pinpoint interaction as a target for development of drugs such as

antagonists.

Structural design of therapeutic molecules can be done at three levels : protein, peptide, and for low molecular weight chemicals. At the protein level, we can change the protein function including the stable agonist or antagonist by protein engineering.[57] At the peptide level, several successful observations are possible. For example, IL-1 receptor and antagonistic peptide complex structures have been studied.[58] This is an important lead compound for drug determinations. For screening of chemicals, we are now performing virtual ligand screening instead of actual activity assay. HTS methods such as FLISA, as described above, will enhance the second step involving the actual screening.

Structural analysis of molecular recognition can help us to discuss disease mechanisms including genotype-phenotype correlations or therapeutic approaches on the scale of drug molecules. This atomic scale strategy should bring with it great contributions to understanding the pathogenesis of allergies, and to develop therapies against allergy (Fig. 7).

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